Primer

Post-translational modifications of tubulin

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Microtubules are the largest filamentous components of the eukaryotic cytoskeleton. In spite of their extraordinary level of structural conservation, microtubules fulfill a vast range of different functions in cells. How this functional diversity is achieved remains an open question; however, recent advances point towards post-translational modifications (PTMs) of tubulin as a potent mechanism to generate microtubule identities. As many microtubule functions have direct implications for development and homeostasis of organisms, understanding the molecular functions of tubulin PTMs could provide a more differentiated view on the role of microtubules in both normal and pathological aspects of organism development.

Microtubules are involved in a vast array of cellular functions, including cell shape, motility and division, intracellular signaling and transport, cell differentiation, and generation of specific organelles. Microtubule functions can be regulated by modulating the biophysical parameters of the microtubules themselves, as well as by their interactions with specific microtubule-associated proteins (MAPs). Strikingly little is known about how specific MAPs can bind selectively to subsets of microtubules inside cells. One possible regulatory mechanism is the spatially and temporally restricted creation of microtubule identities by generating patterns of tubulin PTMs that are commonly referred to as the 'tubulin code'.

Microtubules are assembled from evolutionarily conserved dimers of α- and β-tubulin that can be subjected to a broad range of PTMs. Some of these PTMs are ubiquitous protein modifications, such as acetylation, phosphorylation or palmitoylation, while others are less common, and some appear to be unique to tubulin. Among these rare modifications are the removal of gene-encoded amino acids by detyrosination and the follow-up deglutamylation of α-tubulin, or the addition of amino acids by tyrosination, polyglutamylation, or polyglycylation (Figure 1).

Signals generated by tubulin PTMs can give rise to different levels of information complexity. Simple modifications, such as acetylation, or detyrosination/tyrosination, generate binary signals, while polyglutamylation, polyglycylation, or polyamination can generate more graded signals due to variations in side-chain lengths and modification of either α- or β-tubulin or both. Most importantly, tubulin PTMs can affect different functional roles of microtubules depending on the localization of these modification sites. Acetylation, for instance, is found at the luminal surface of the microtubules, while most other tubulin PTMs modify the carboxy-terminal tails of tubulin that are located at the outer surface of microtubules (Figure 1). As an important interaction site for many MAPs, the carboxy-terminal tail is thought to be a hotspot for the selective regulation of microtubule–MAP interactions. In this Primer, we discuss the various tubulin PTMs and their effects on microtubule functions.

Acetylation

To date, the most-studied tubulin modification is acetylation of lysine 40 (K40) of α-tubulin. The peculiarity of this modification site is its position at the luminal surface of microtubules. This feature renders it unlikely to regulate the binding of MAPs and motors to the outer surface of microtubules, while it is more likely to influence the binding of luminal proteins (Figure 1). The anti-K40-acetylation antibody stains discrete microtubule populations in interphase cells and reveals that ciliary, flagellar and neuronal microtubules are strongly acetylated. K40 acetylation is catalyzed by the acetyl transferases αTAT/Mec-17 and Atat-2 (Atat-2 has so far only been found in *Caenorhabditis elegans*), and deacetylation is performed by the deacetylases HDAC6 and SIRT2 (Figure 2). Little is known about the functions of K40 acetylation. Initial observations suggesting a regulation of kinesin motors by microtubule acetylation at K40 could not be confirmed by later studies. In *C. elegans*, K40 acetylation is required in touch receptor neurons, and a recent study has shown that the modification is part of a mechanism that orients cells during three-dimensional migration.

A second acetylation event has recently been found on K252 of β-tubulin (Figure 1); this acetylation may negatively regulate microtubule assembly. Acetylation of K252 is catalyzed by the Sun acetyltransferase (Figure 2), which, in contrast to αTAT (the α-tubulin K40 acetylase) modifies free tubulin dimers and negatively regulates their assembly into microtubules. The possibility that tubulins are subject to more complex acetylation events was evoked by the identification of a number of potential acetylation sites on both α- and β-tubulin in a whole-proteome mass spectrometry study. None of the sites identified in this study has so far been confirmed by cell biological or biochemical approaches, yet some of these sites are especially intriguing because they are localized at the boundaries between α- and β-tubulin subunits and could thus have an important impact on microtubule assembly.

Detyrosination/tyrosination

The enzymatic, ribosome- and tRNA-independent incorporation of tyrosine into α-tubulin was discovered in 1973 and shown to be reversible in 1977. Sequencing of the α-tubulin genes at the end of the 1970s revealed that tyrosine is actually encoded by the α-tubulin gene — thus, the initial modification is detyrosination. Amazingly, the enzyme catalyzing detyrosination has so far not been discovered, while the enzyme catalyzing the reverse reaction, tubulin tyrosine ligase (TTL; Figure 2), was the first tubulin-modifying enzyme to be identified. Biochemical and structural work has demonstrated that TTL exclusively modifies unpolymerized tubulin, which implies that the detyrosination/retyrosination cycle of tubulin depends critically upon the dynamic instability of microtubules. This mechanism, together with the preference of the tubulin-detyrosinating enzyme for polymerized microtubules, results in an accumulation of detyrosinated tubulin in stable microtubules.

Removal of the carboxy-terminal tyrosine from the α-tubulin protein exposes the penultimate glutamate residue (Figure 1), which is the

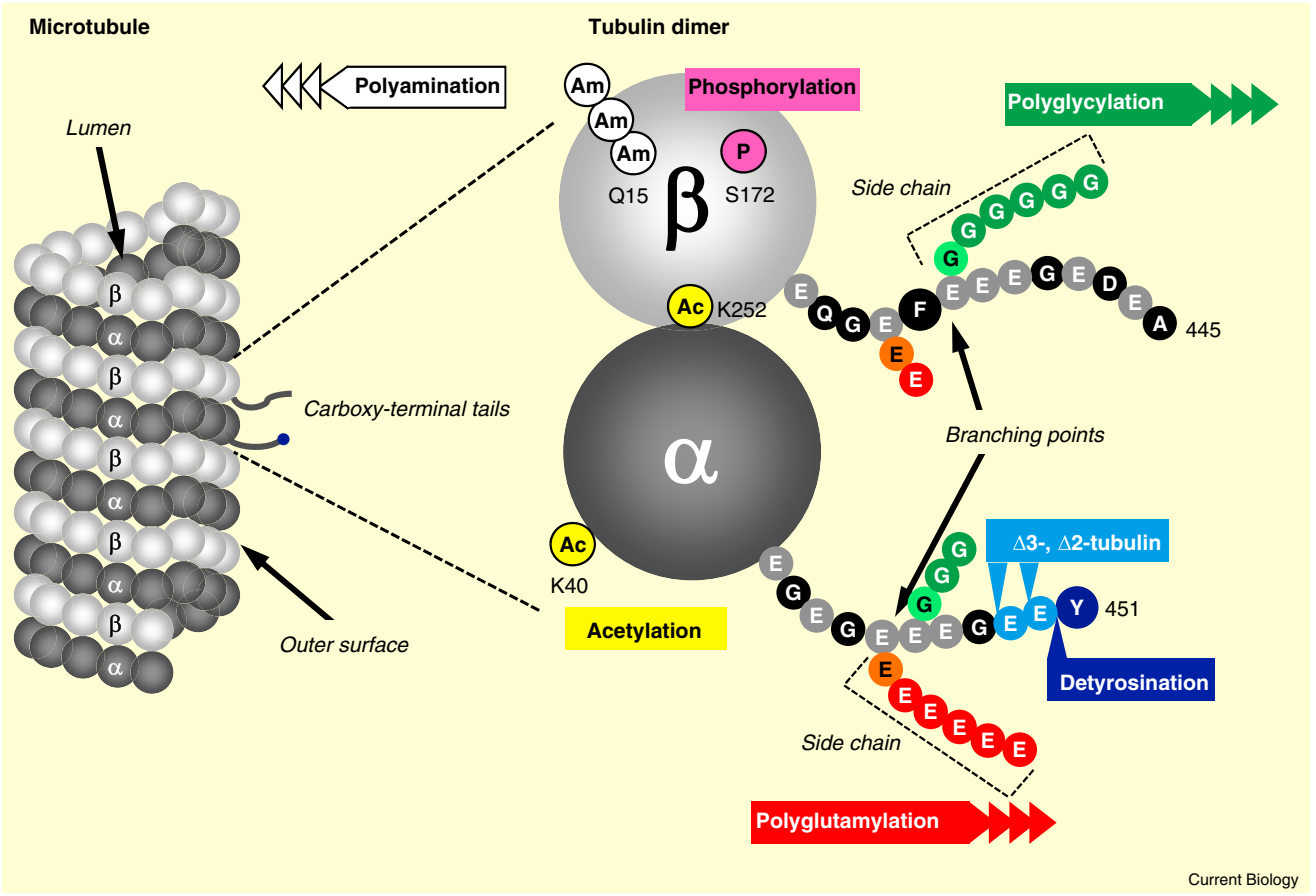

Figure 1. Molecular localization of tubulin posttranslational modifications.
Microtubules are assembled from α-tubulin–β-tubulin dimers, which form hollow tubes composed of 13 protofilaments. The globular parts of the tubulins form the microtubule walls and the luminal surface, while the carboxy-terminal tails decorate the outer surface of microtubules, where many MAPs and motors bind. PTMs are found on various regions of the tubulin dimer: the α-tubulin K40 acetylation site is present at the luminal surface of microtubules, while the K252 acetylation site of β-tubulin is present on the boundary between α- and β-tubulins. Polyamination at Q15 and phosphorylation at S172 are found in the globular, folded part of β-tubulin and might influence the assembly rates and stability of microtubules. Detyrosination/tyrosination, Δ2- and Δ3-tubulin modulate the carboxy-terminal tails of α-tubulins, and polyglutamylation and polyglycylation are found within the carboxy-terminal tails of both α- and β-tubulin. All modifications of the carboxy-terminal tails are likely to regulate interactions between microtubules and associated proteins.

genesis of the original nickname for detyrosinated tubulin, 'Glu-tubulin'. This nickname has led to some confusion, however, since the discovery of tubulin glutamylation; detyrosinated tubulin is now usually called detyr-tubulin. Tyrosinated tubulin is consistently called tyr-tubulin.

In most organisms studied to date, only one TTL enzyme is present in the genome, and removal of this enzyme in mice led to a huge increase in detyr-tubulin (and Δ2-tubulin; see next section) in cells. Moreover, *TTL*-knockout mice die at the perinatal stage due to neuronal abnormalities, which most likely result from mislocalization of the microtubule plus-end tracking protein (+TIP) CLIP170. The function of detyrosination is linked to its ability to modulate microtubule–MAP interactions: detyrosination enhances kinesin-1-driven transport in neurons, while it inhibits the microtubule plus-end localization of a subgroup of +TIPs — the CAP-Gly domain proteins. Moreover, detyrosination negatively regulates the activity of MCAK, a microtubule-depolymerizing kinesin. Detyrosinated tubulin is present at high levels in neuronal microtubules and other long-lived microtubule populations, but a direct causal relationship between detyrosination and microtubule stability has not been established.

The tyrosination status of microtubules has many functional implications. It is important for processes that depend on +TIP functions and can thus influence spindle orientation or growth cone guidance in neuronal pathfinding. Tyrosination levels also regulate microtubule dynamics and turnover in cells. The impact of detyrosination on kinesin-1 traffic, though subtle, could have a huge impact on neuronal transport due to the great distances that need to be covered in axons.

A pathological side effect of the detyrosination/retyrosination cycle could be the incorporation of nitrotyrosine into tubulin. Indeed, in a number of human disorders, the presence of nitric oxide leads to the nitration of free cellular tyrosine, which can be incorporated into α-tubulin by TTL. In contrast to tyr-tubulin, nitrotyrosinated tubulin cannot be detyrosinated, thus impacting on the balance between tyr- and detyr-tubulin, and potentially on the functions of the affected microtubules.

Δ2- and Δ3- tubulin

Following detyrosination, the carboxy-terminal tail of α-tubulin can be further modified by the removal of the penultimate glutamate residue, generating Δ2-tubulin (Figure 1). This deglutamylation reaction is catalyzed by enzymes from the cytosolic carboxypeptidase (CCP) family, which are also involved in the removal of post-translationally added polyglutamylation (Figure 2; see below). Continued proteolysis of the carboxy-terminal tubulin tail generates Δ3-tubulin, which suggests that further carboxy-terminal degradation of tubulin tails might be possible (Figure 1). Δ2-tubulin cannot be retyrosinated, thus irreversibly locking tubulin in the non-tyrosinatable status, which could be one of the possible functions of this PTM. It is tempting to speculate that further degradation of tubulin tails could remove the carboxy-terminal modification sites for other PTMs, such as polyglutamylation and polyglycylation.

Polyglutamylation

Polyglutamylation is the enzymatic addition of side chains of glutamates onto gene-encoded glutamate residues of the modified proteins (Figure 1). In α- and β-tubulins, polyglutamylation occurs on several sites within the carboxy-terminal tails, and side chains of various lengths are generated. The identification of the glutamylating enzymes revealed that the final polyglutamylation patterns on microtubules are determined by the activities of enzymes involved in the modification. Polyglutamylation is catalyzed by members of the tubulin tyrosine ligase-like (TTLL) family, each of which has a particular reaction preference — α- vs. β-tubulin and initiation vs. elongation of the glutamate side chains. Polyglutamylation is a reversible modification, and enzymes removing glutamate side chains belong to the cytosolic carboxypeptidase (CCP) family. Similar to the glutamylases, some deglutamylases preferentially shorten long glutamate chains, whereas other enzymes can specifically remove the branching point of glutamate side chains (Figure 2).

Microtubule polyglutamylation affects the charges on the carboxy-terminal tails of tubulins and is thus believed to regulate electrostatic microtubule–MAP interactions. The first regulatory mechanism to be identified for microtubule polyglutamylation is microtubule-severing catalyzed by the enzyme spastin, the activity of which is much greater on polyglutamylated than on non-modified microtubules. This mechanistic study indicates that the mass and length of microtubules in cells may be regulated by subtle changes in microtubule polyglutamylation; also, preferred microtubule-severing sites might be determined by modification marks. Other proteins likely to be regulated by glutamylation are molecular motors and MAPs that bind specifically to the carboxy-terminal tubulin tails, but no experimental support for this is thus far available.

Glutamylation can be found in many cell types and on many cellular microtubule subsets; however, modification levels vary markedly. Particularly high levels have been found on centrioles, on the axonemes of cilia and flagella, and in neurons. During cell division, glutamylation levels are temporarily increased on the central mitotic spindle and on the midbody. Hyperglutamylation has been linked to neurodegeneration in a mouse model for Purkinje cell degeneration (*pcd*), underscoring the importance of balanced levels of microtubule polyglutamylation for neurons. Moreover, polyglutamylation has been reported to regulate the beating of motile cilia in different model organisms. Depletion of selected polyglutamylases in the protists *Chlamydomonas reinhardtii* and *Tetrahymena thermophilia* and

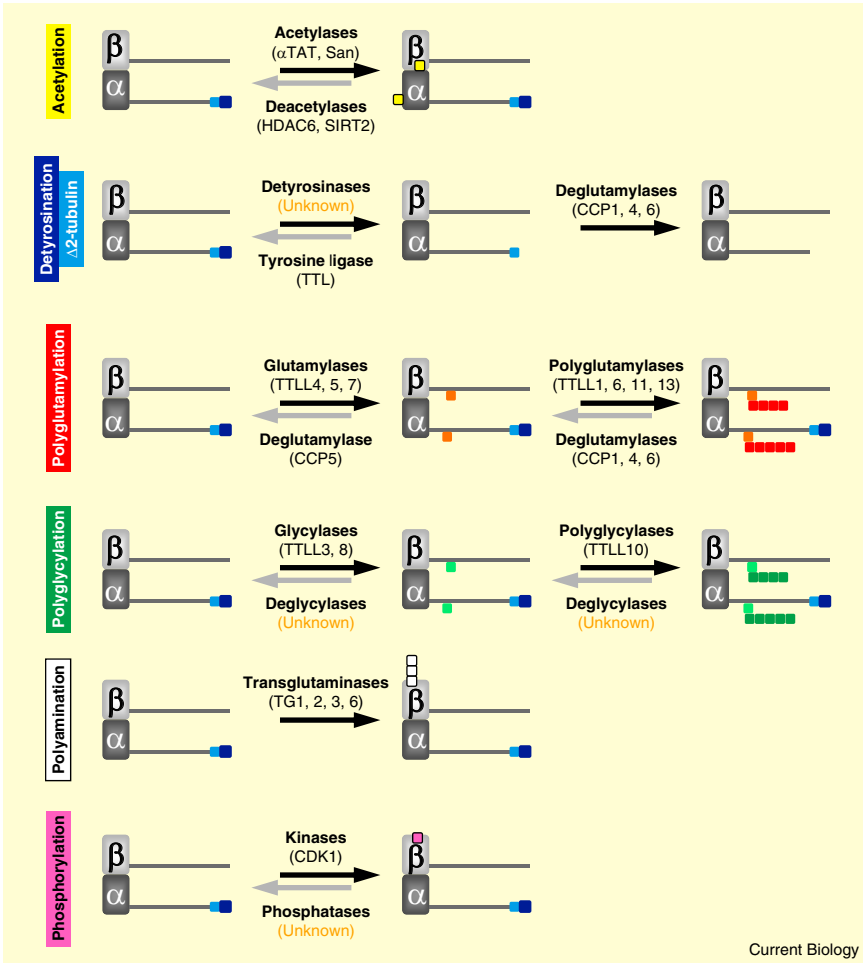

Figure 2. Enzymes involved in tubulin post-translational modifications.
Schematic representation of the known enzymes responsible for tubulin PTMs. The forward reactions (modifications) take place on microtubules, while the reverse reactions (demodifications) mostly affect soluble tubulin. Note that some reactions are irreversible, and some enzymes have not yet been identified. αTAT, α-tubulin N-acetyltransferase; HDAC6, histone deacetylase 6; SIRT2, sirtuin 2; TTL, tubulin tyrosine ligase; TTLL, TTL-like; CCP, cytosolic carboxypeptidase; TG, transglutaminase; CDK1, cyclin-dependent kinase 1. The color code corresponds to Figure 1.

in mouse ependymal cells led to a severe perturbation of ciliary beating, although cilia did not disassemble. Thus, polyglutamylation, and even further, specific polyglutamylation enzymes, are required for proper regulation of flagellar dynein and thus ciliary beating. The particularly high polyglutamylation levels found on mammalian centrioles have also been suggested to stabilize centrosomes, especially when external forces are exerted during cell division.

Polyglycylation

Polyglycylation is a tubulin modification analogous to polyglutamylation: it creates side chains of glycine, potentially using the same acceptor glutamate residues on target proteins. Glycylation modifies both α- and β-tubulins, has several modification sites within the carboxy-terminal tubulin tails, and generates chains of different lengths (Figure 1). Glycylases are members of the TTLL family and, as for glutamylases, in mammals they can be subdivided into initiating and side-chain-elongating glycylases (Figure 2). However, in *Drosophila* the enzymes are multifunctional, performing both initiation and elongation.

To date, glycylation has been found exclusively in axonemes of cilia and flagella, and little is known about its function in mammals. Glycylation has been shown to be crucial for the stability and maintenance of axonemes in *Drosophila melanogaster* sperm tails, which, in the absence of the testis-specific glycylase enzyme, disassemble completely during the maturation process. Similarly, co-depletion of the two initiating glycylases in mouse ependymal cells leads to a complete disassembly of motile cilia. Strikingly, while glycine side chains are elongated to generate polyglycylation in most organisms, the essential functions of the poly-modification seem to be sufficiently fulfilled by short glycine side chains. Curiously, although present in other primates, the enzymatic ability to generate polyglycylation is absent in humans as a result of two specific amino acid changes within the human genome.

Polyamination

Tubulin polyamination is a recently described irreversible PTM involving the addition of amines to glutamine residues of tubulin (Figure 1). The enzyme responsible for tubulin polyamination is a transglutaminase that can polyaminate both free tubulin and microtubules (Figure 2). A biochemical peculiarity of this modification is the fact that it adds positive charges to the overall acidic tubulin.

The amination modification was overlooked until recently because, in the classical tubulin purification protocol, aminated tubulin is excluded. Polyaminated microtubules are highly stable in cold- or calcium-induced depolymerization conditions, and thus polyamination is believed to contribute to the stability of microtubule subpopulations in neurons. Consistently, polyamination sites are found either close to the GTP-binding pocket of β-tubulin, or at the α-tubulin–β-tubulin dimer boundary.

Phosphorylation

Early biochemical studies have shown that tubulin can be phosphorylated, although only one precise phosphorylation site has been functionally analyzed so far. Phosphorylation of the serine residue S172 of β-tubulin has been shown to influence microtubule dynamics (Figure 1). This phosphorylation is catalyzed by cyclin-dependent kinase 1 (CDK1; Figure 2), and thus has been suggested to regulate microtubule behavior during cell division. Another intriguing, but less fully characterized, phosphorylation event is catalyzed by the tyrosine kinase Syk, which phosphorylates an unidentified residue within the carboxy-terminal domain of α-tubulin. The position of the modification site is such that it may affect binding of MAPs to microtubules; however, no functional data have yet demonstrated this.

Other modifications

Several other PTMs have been identified on tubulin, but little functional insight has been gained, and follow-up studies have yet to be performed. For example, ubiquitination of tubulin has been implicated in the proteolytic degradation of misfolded tubulin in cells; and tubulin palmitoylation has been suggested to regulate microtubule–membrane interactions. Tubulin glycosylation, arginylation, methylation and sumoylation have also been reported, but no details of sites or relevant functions have been provided so far.

Perspectives

Taken together, multiple and complex tubulin PTMs provide a myriad of combinatorial possibilities to specifically 'tag' microtubule subpopulations in cells, thus destining them for precise functions. How this tubulin or microtubule code allows cells to divide, migrate, communicate and differentiate in an ordered manner is an exciting question that needs to be answered in the near future. Initial insights have already revealed the potential roles of tubulin PTMs in a number of human pathologies, like cancer, neurodegeneration and ciliopathies.

Further reading

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